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Hakomori

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[45] **Date of Patent: Feb. 14, 1995**

[54] **METHODS FOR THE PRODUCTION OF ANTIBODIES AND INDUCTION OF IMMUNE RESPONSES TO TUMOR-ASSOCIATED GANAGLIOSIDES BY IMMUNIZATION WITH GANGLOISIDE LACTONES**

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[21] Appl. No.: **97,006**

[22] Filed: **Jul. 27, 1993**

Related U.S. Application Data

[60] Division of Ser. No. 996,509, Dec. 21, 1992, Pat. No. 5,308,614, which is a continuation of Ser. No. 173,962, Mar. 28, 1988, abandoned.

[51] **Int. Cl.**[6] **C07K 15/28;** C12P 21/08; C12N 15/02

[52] **U.S. Cl.** **435/70.21;** 435/70.2; 435/172.2; 435/240.23; 530/388.8; 530/388.85; 530/809; 530/867

[58] **Field of Search** 435/172.2, 70.2, 7.23; 424/88; 530/388.8, 388.85; 436/512, 548

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Assistant Examiner—Thomas Cunningham
Attorney, Agent, or Firm—Sughrue, Mion, Zinn, Macpeak & Seas

[57] **ABSTRACT**

The present invention relates to an improved method for the production of antibodies to tumor-associated gangliosides using ganglioside lactones. The resulting antibodies are useful in the detection and treatment of tumors containing gangliosides. The present invention also relates to methods of treatment of tumors by active immunization using ganglioside lactones.

5 Claims, 9 Drawing Sheets

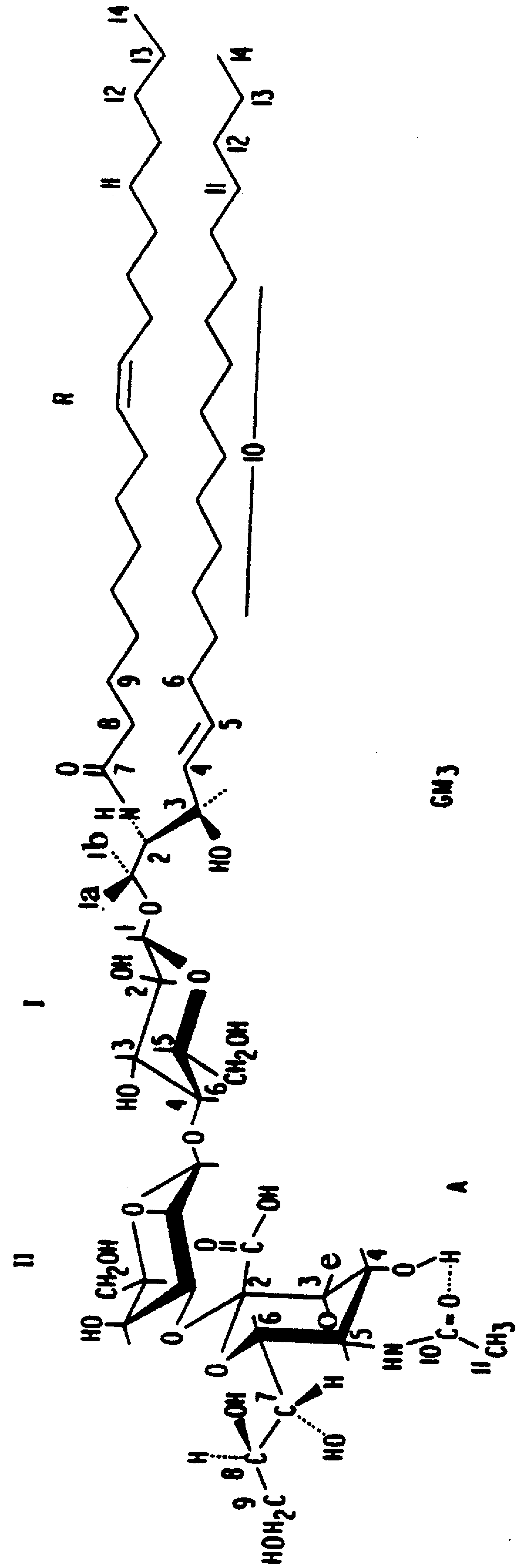
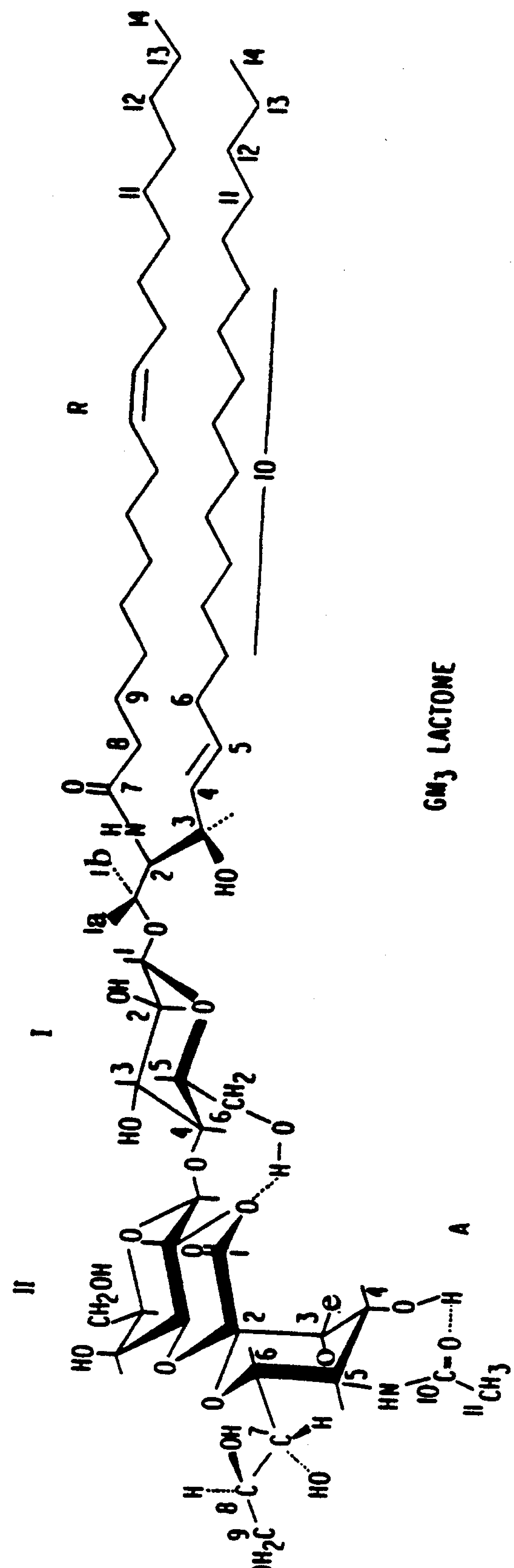


FIG.1A



GM$_3$ LACTONE

FIG. 1B

FIG. 2A

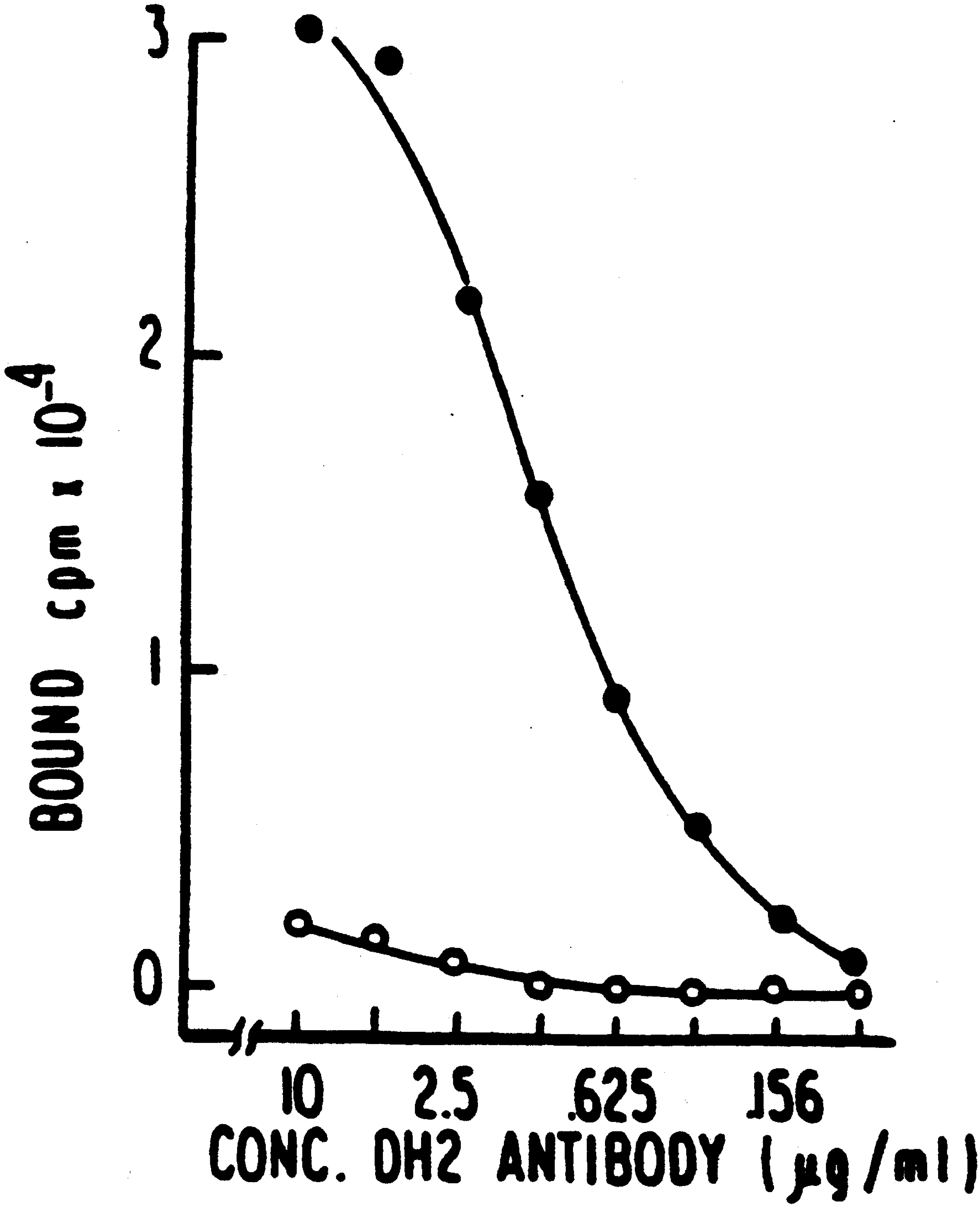
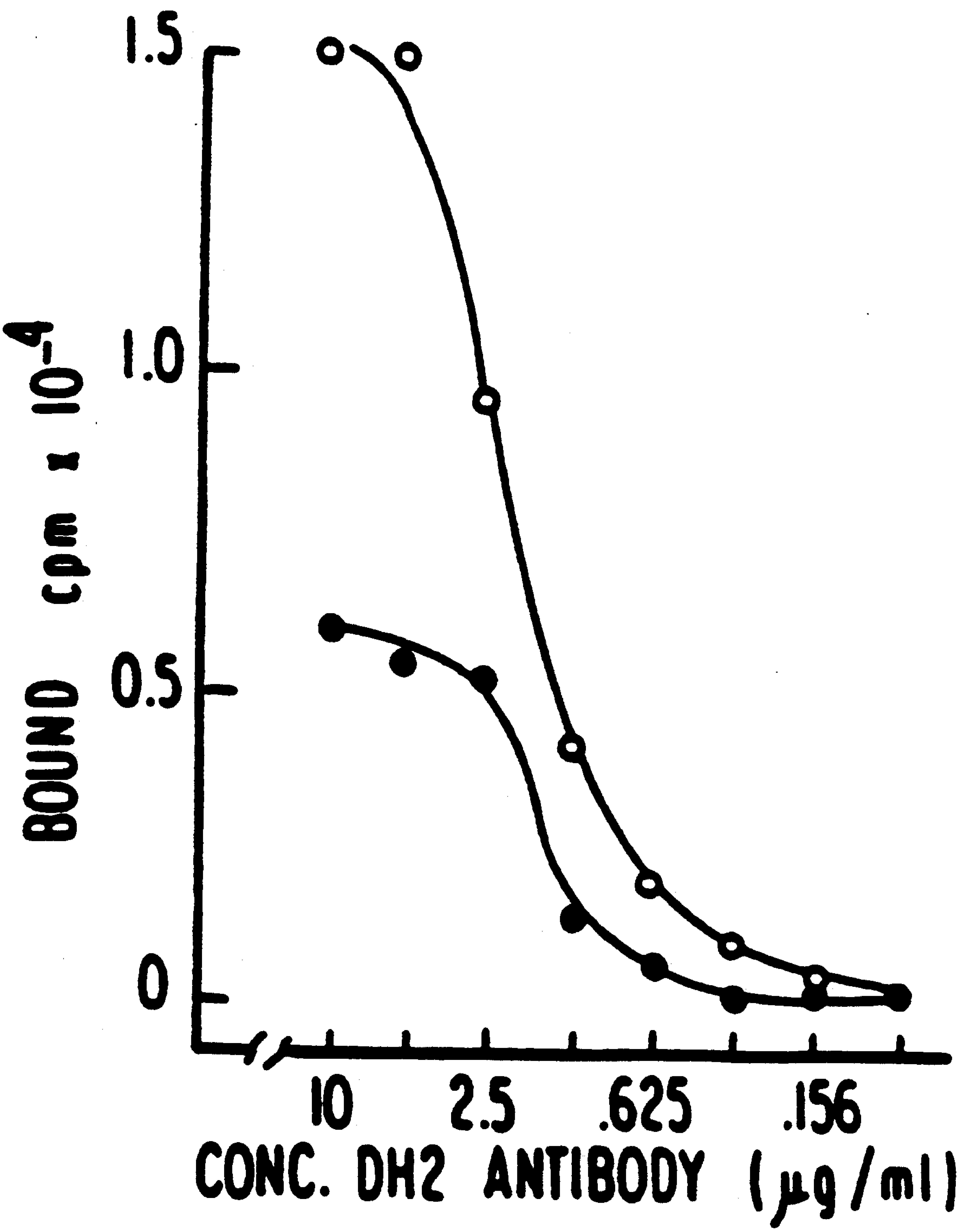


FIG. 2B



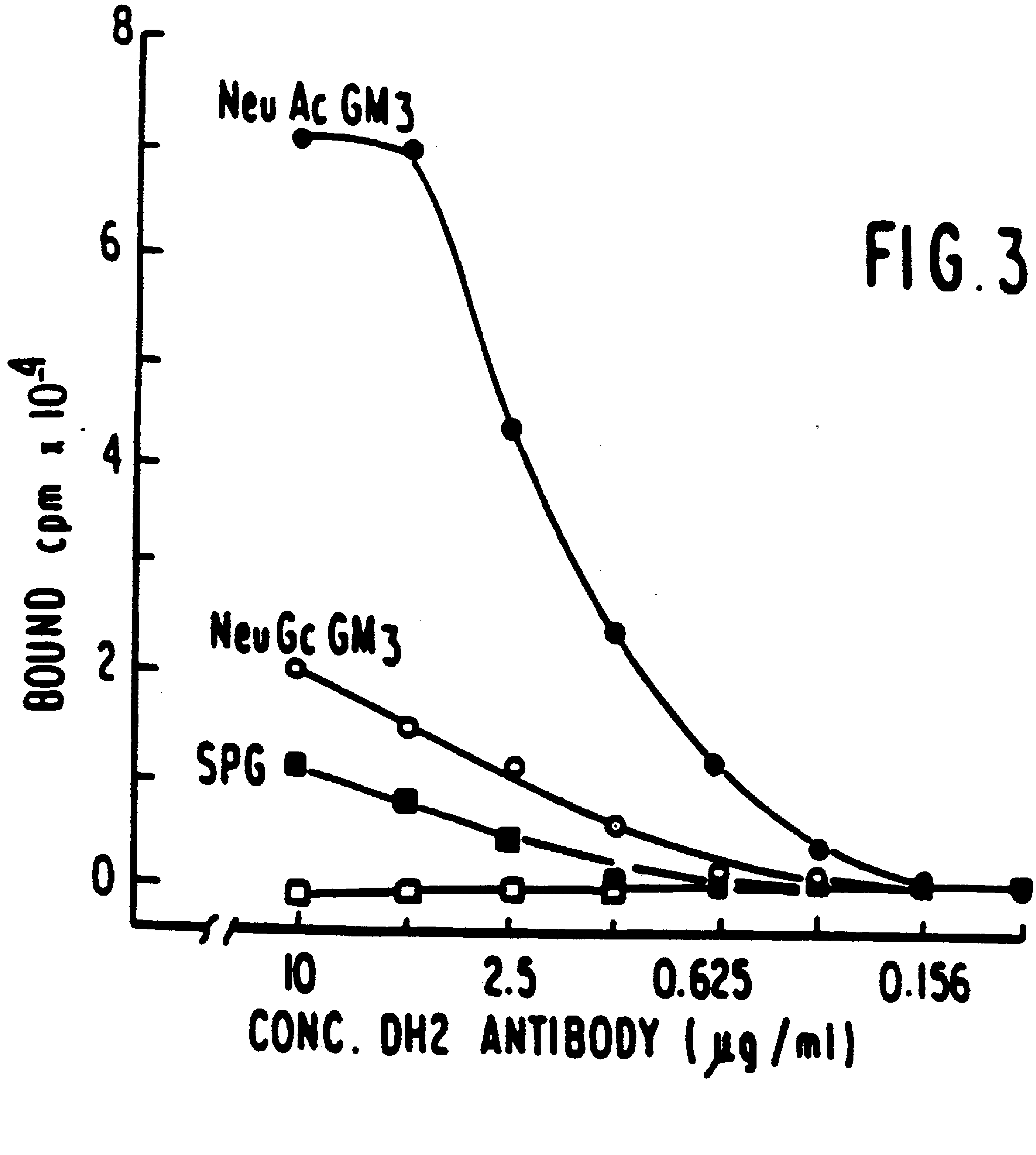

8
6
4
2
0
BOUND cpm x 10^{-4}
Neu Ac GM3
Neu Gc GM3
SPG
10
2.5
0.625
0.156
CONC. DH2 ANTIBODY (µg/ml)

FIG. 3

FIG. 4

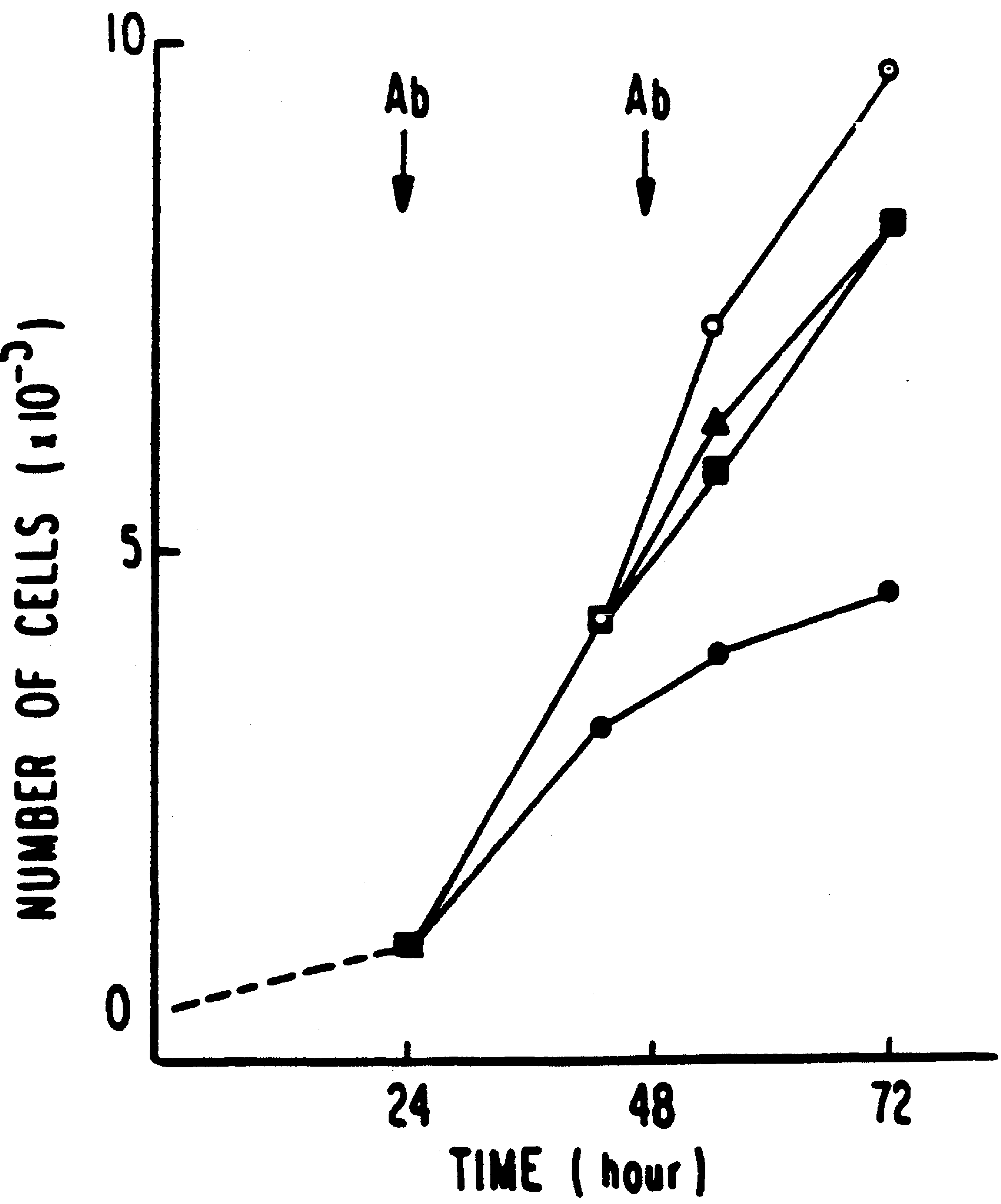


FIG.5

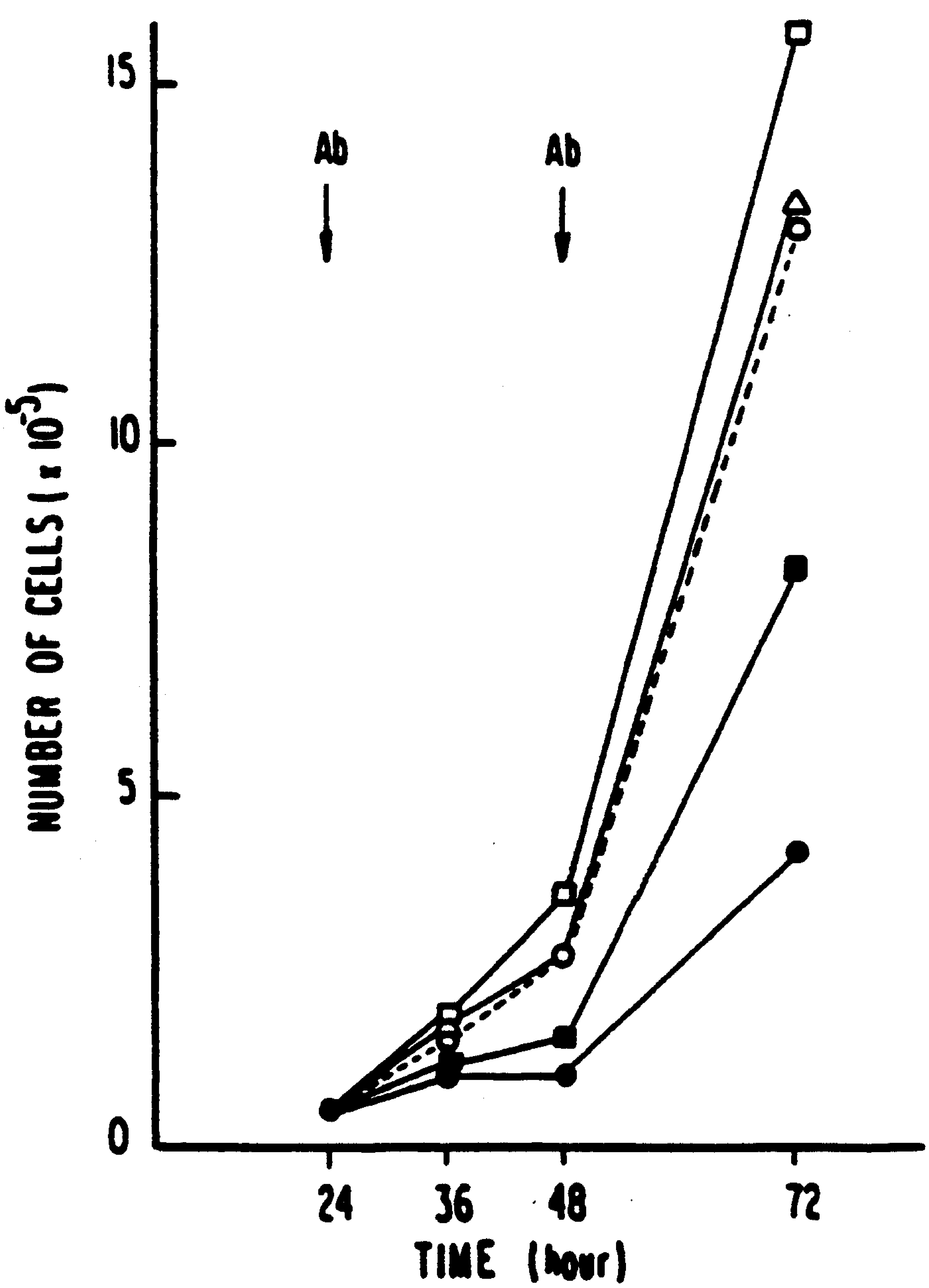
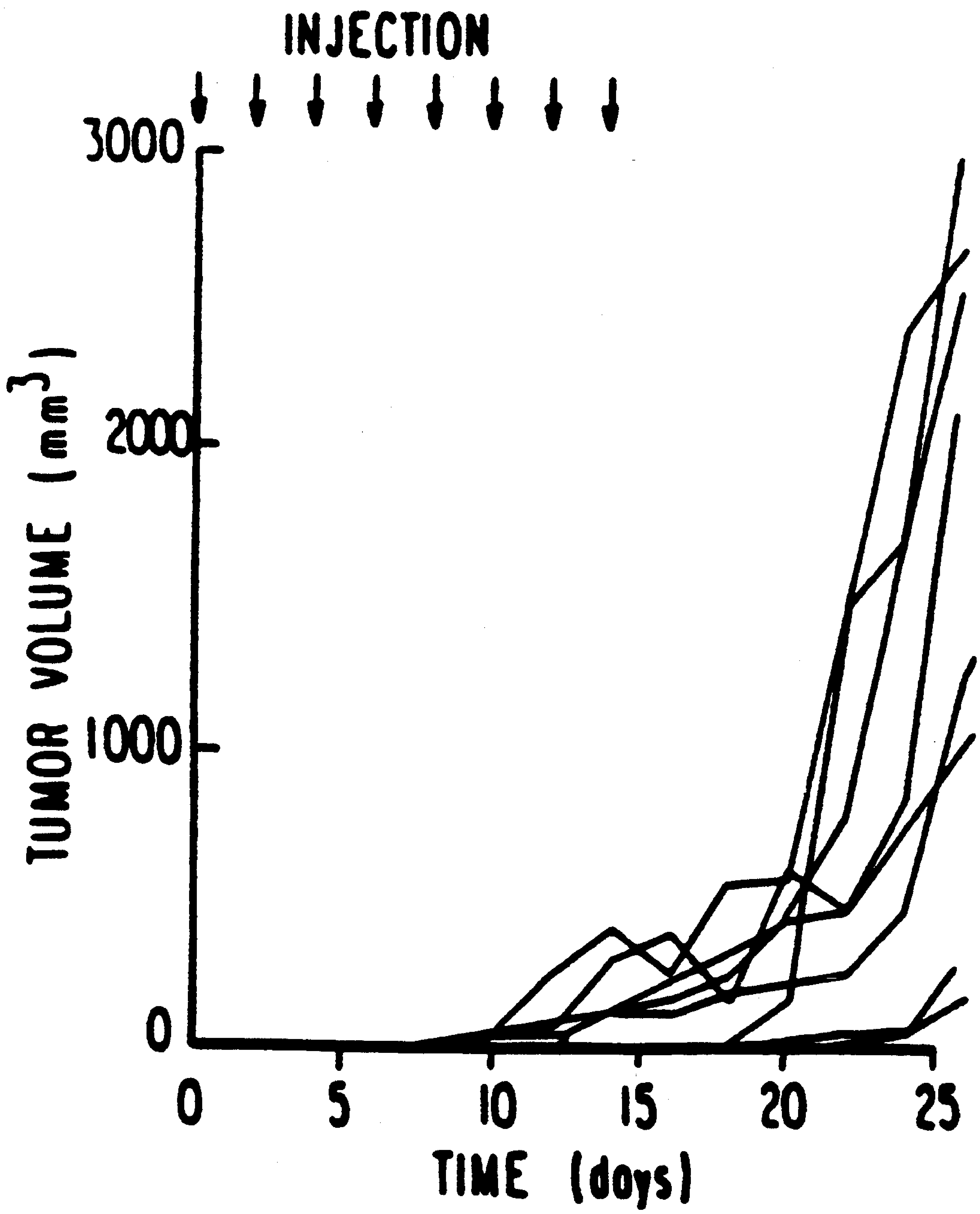


FIG.6A


INJECTION
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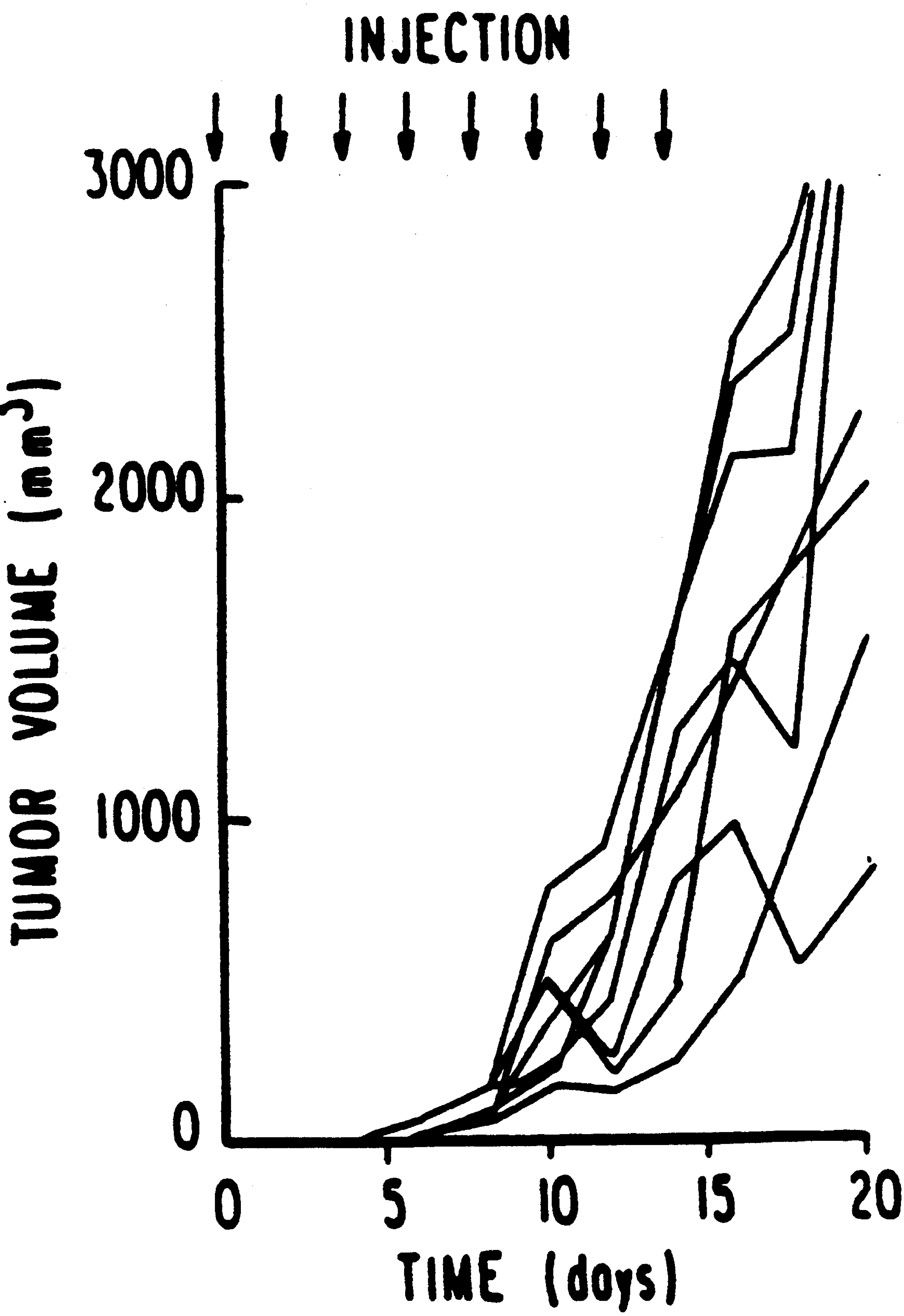
2000
1000
0
TUMOR VOLUME (mm3)
0
5
10
15
20
25
TIME (days)

FIG. 6B


INJECTION
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TUMOR VOLUME (mm³)
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TIME (days)

METHODS FOR THE PRODUCTION OF ANTIBODIES AND INDUCTION OF IMMUNE RESPONSES TO TUMOR-ASSOCIATED GANAGLIOSIDES BY IMMUNIZATION WITH GANGLOISIDE LACTONES

This is a divisional of application Ser. No. 07/996,509 filed Dec. 21, 1992, now U.S. Pat. No. 5,308,614, which is a continuation of application Ser. No. 07/173,962, filed Mar. 28, 1988, now abandoned.

FIELD OF THE INVENTION

The present invention relates to an improved method for the production of antibodies to tumor-associated gangliosides using ganglioside lactones. The resulting antibodies are useful in the detection and treatment of tumors containing gangliosides. The present invention also relates to methods of treatment of tumors by active immunization using ganglioside lactones.

BACKGROUND OF THE INVENTION

Cells are surrounded by plasma membranes. Plasma membranes contain components called glycosphingolipids inserted therein which aide in the formation of the characteristic surface structure of the cells. Each type of cell is characterized by a specific profile of the glycosphingolipid components, including those components known as gangliosides, located in its plasma membrane. Gangliosides contain a particular type of acidic carbohydrate known as sialic acid. Further, many specific types of cells, including tumor cells, are characterized by the presence of a particular type of ganglioside located in their plasma membranes.

In recent years, a number of monoclonal antibodies have been established after immunization with human tumor cells or tissues. These monoclonal antibodies were selected by their positive reactivity to tumor cells and negative reactivity to normal cells or tissues. Many of the monoclonal antibodies selected by preferential reactivity to melanomas, neuroblastomas and adenocarcinomas have been identified as being directed to gangliosides. Some of these anti-ganglioside antibodies with specific isotopes (particularly IgG_3 and IgG_{2a}) and which show strong reactivity to gangliosides, have been found to suppress tumor growth in vivo. For example, melanomas of some patients have been found to regress following a large dose administration of a specific anti-GD_3 ganglioside antibody (Houghton, A. N. et al, *Proc. Natl. Acad. Sci. USA*, 82:1242-1246 (1985)). Further, recently it has been demonstrated that GM_2 absorbed on BCG bacteria showed a detectable immune response. Thus, it has been asserted that GM_2 could be a useful vaccine for human melanomas (Livingston, P.O. et al, *Proc. Natl. Acad. Sci. USA*, 84:2911-2915 (1987)). Hence, gangliosides are important antigens and immunogens of tumor tissues and cells (Hakomori, S., *Annu. Rev. Immunol.*, 2:103-126 (1984); Hakomori, S., In *Handbook of Lipid Research*, Volume 3, *Sphingolipid Biochemistry*, Kanfer, J. N. et al Eds., Plenum, N.Y., pages 1-165 (1983); and Hakomori, S., *Sci. Amer.*, 254:44-53 (1986)).

However, the use of tumor cells (including cell membranes), tumor tissues, or isolated gangliosides absorbed on bacteria as immunogens, is extremely laborious and requires extensive selection studies. In addition, although gangliosides are important cell type-specific markers, they are poor immunogens in eliciting humoral or cellular immune responses. As a result, repeated immunization with tumor cells (including cell membranes), tumor tissues or isolated gangliosides absorbed on bacteria or other carriers is disadvantageously necessary.

A small portion of gangliosides are present in tumor cells and tissues in the form of a lactone thereof. For example, less than 0.1% of the particular ganglioside, designated GM_3 (see FIG. 1A), present in melanoma cells has been identified as a lactone thereof (see FIG. 1B). Ganglioside lactones are defined as the inner ester between the carboxyl group of the sialic acid and the primary or secondary hydroxyl group of the sugar residues within the same molecule. One example of a GM_3 lactone, wherein the carboxyl group of sialic acid is esterified with the C-2 secondary hydroxyl group of the penultimate galactose is shown in FIG. 1B (Yu, R. K. et al, *J. Biochem. Tokyo*, 98:1307 (1985)). This structure is sterically stable and relatively stable at acidic to neutral pH, although unstable at alkaline pH. While galactoside lactones have been detected and believed to be naturally occurring plasma membrane components, their quantity is extremely low and thus their natural occurrence has been disputed (Nores, G. A. et al, *J. Immunol.*, 139:3171-3176 (1987) and Riboni, L., *J. Biol. Chem.*, 261:8514-8519 (1986)).

Despite the question about their natural occurrence, it has been demonstrated in the present invention that ganglioside lactones are strong immunogens, which can cause a much greater immune response than native gangliosides. Further, it has been found in the present invention that the antibodies produced using ganglioside lactones as immunogens are of the IgG_3 isotype, which is extremely useful, compared to antibodies of the IgM isotype produced using native gangliosides, (i) in detecting tumors containing gangliosides, (ii) in suppressing growth of tumors containing gangliosides in vitro and in vivo and (iii) in inducing antibody-dependent cytotoxicity in vivo. In addition, it has been found in the present invention that ganglioside lactones themselves are effective for suppressing growth of tumors containing gangliosides in vivo, whereas such suppression is not achieved using native gangliosides.

SUMMARY OF THE INVENTION

Accordingly, an object of the present invention is to provide a method for the production of antibodies to tumor-associated gangliosides.

Another object of the present invention is to provide a passive immunization method for treating tumors containing gangliosides.

Still another object of the present invention is to provide an active immunization method for treating tumors containing gangliosides.

Yet another object of the present invention is to provide a method for detecting tumors containing gangliosides.

These and other objects of the present invention, which will be apparent from the detailed description of the invention provided hereinafter, have been met by the following embodiments.

In one embodiment, the present invention relates to a method for the production of antibodies to tumor-associated gangliosides comprising:

(1) immunizing an animal with an immunogenic effective amount of a lactone of a tumor-associated ganglioside and a pharmaceutically acceptable carrier;
(2) isolating the immunized cells from said animal;

(3) fusing the isolated immunized cells with myeloma cells; and
(4) screening for hybridomas which produce antibodies having binding specificity to said ganglioside and collecting the antibodies so produced.

In a second embodiment, the present invention relates to a passive immunization method for treating tumors containing gangliosides comprising administering to a subject:

(A) a pharmaceutically effective amount of an antibody produced by the process comprising:
 (1) immunizing an animal with an immunogenic effective amount of a lactone of a tumor-associated ganglioside and a pharmaceutically acceptable carrier;
 (2) isolating the immunized cells from said animal;
 (3) fusing the isolated immunized cells with myeloma cells; and
 (4) screening for hybridomas which produce antibodies having binding specificity to said ganglioside and collecting the antibodies so produced; and
(B) a pharmaceutically acceptable carrier.

In a third embodiment, the present invention relates to an active immunization method for treating tumors containing gangliosides comprising administering to a subject:

(A) an immunogenic effective amount of a lactone of a tumor-associated ganglioside; and
(B) a pharmaceutically acceptable carrier.

In a fourth embodiment, the present invention relates to a method for detecting tumors containing gangliosides comprising:

(A) contacting a test sample with an antibody produced by the process comprising:
 (1) immunizing an animal with an immunogenic effective amount of a lactone of a tumor-associated ganglioside and a pharmaceutically acceptable carrier;
 (2) isolating the immunized cells from said animal;
 (3) fusing the isolated immunized cells with myeloma cells;
 (4) screening for hybridomas which produce antibodies having binding specificity to said ganglioside and collecting the antibodies so produced; and
(B) assaying for specific binding of said antibody to antigen in said test sample.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A illustrates the structure of GM_3. FIG. 1B illustrates the structure of GM_3 lactone. The carboxyl group (COOH) in FIG. 1A and the hydroxyl group at the C-2 position of the galactose (residue II) are esterified to form a six-member ring between the galactose (residue II) and sialic acid (residue A) to give rise to the structure of the GM_3 lactone shown in FIG. 1B. In FIGS. 1A and 1B, residue I and residue R are glucose and ceramide, respectively.

FIG. 2A shows the reactivity of DH2 antibody with GM_3 (.) or GM_3 lactone (o), as determined in a solid-phase radioimmunoassay, which was carried out by dissolving the gangliosides together with phosphatidylcholine (hereinafter "PC") and cholesterol in ethanol and drying on polyvinyl plastic plates. FIG. 2B shows the reactivity of DH2 antibody with GM_3 (.) or GM_3 lactone (o), as determined in a solid-phase radioimmunoassay, which was carried out in gelatin-coated polyvinyl plastic plates on which an aqueous solution of GM_3 or GM_3 lactone was added and incubated to ensure adsorption of such to the gelatin coat. The aqueous solution is made in phosphate buffered saline (hereinafter "PBS").

The data in FIGS. 2A and 2B is the average of triplicate experiments. Note, as shown in FIG. 2B, GM_3 lactone reactivity of DH2 antibody can be specifically detected when adsorbed on gelatin-coated polyvinyl plastic plates.

FIG. 3 illustrates the reactivity of DH2 antibody with the following glycolipids: NeuAcGM_3 (.); NeuGcGM_3 (o); sialylparagloboside (•); or other glycolipids, GM_1, GD1a, GD1b, GT1, galactosylceramide and sialyllactonorhexaosylceramide (all indicated as □), as determined in a solid-phase radioimmunoassay, which was carried out by dissolving the glycolipids together with PC and cholesterol in ethanol and drying in polyvinyl plastic plates. The data in FIG. 3 is the average of triplicate experiments.

FIG. 4 illustrates the inhibition of B16 melanoma cell growth in vitro by the following antibodies: DH2 (.); M2590(▲); Cu-1 anti-Tn (■); and PBS as a control (o). In FIG. 4, each data point represents the average of triplicate experiments. The standard deviation was less than 15%.

FIG. 5 illustrates the effects of the following concentrations of DH2 antibody on B16 melanoma cell growth in vitro: 100 μg/ml (.); 50 μg/ml (■); 25 μg/ml (o); and 12.5 μg/ml (Δ); and PBS as a control (□). In FIG. 5, each data point represents the average of triplicate experiments. The standard deviation was less than 15%.

FIG. 6A illustrates the effect of DH2 antibody on B16 melanoma growth in vivo. FIG. 6B illustrates the effect of PBS as a control on B16 melanoma growth in vivo.

DETAILED DESCRIPTION OF THE INVENTION

As discussed above, in one embodiment, the present invention relates to a method for the production of antibodies to tumor-associated gangliosides comprising:

(1) immunizing an animal with an immunogenic effective amount of a lactone of a tumor-associated ganglioside and a pharmaceutically acceptable carrier;
(2) isolating the immunized cells from said animal;
(3) fusing the isolated immunized cells with myeloma cells; and
(4) screening for hybridomas which produce antibodies having binding specificity to said ganglioside and collecting the antibodies so produced.

In a second embodiment, the present invention relates to a passive immunization method for treating tumors containing gangliosides comprising administering to a subject:

(A) a pharmaceutically effective amount of an antibody produced by the process comprising:
 (1) immunizing an animal with an immunogenic effective amount of a lactone of a tumor-associated ganglioside and a pharmaceutically acceptable carrier;
 (2) isolating the immunized cells from said animal;
 (3) fusing the isolated immunized cells with myeloma cells; and
 (4) screening for hybridomas which produce antibodies having binding specificity to said ganglioside and collecting the antibodies so produced; and
(B) a pharmaceutically acceptable carrier.

In a third embodiment, the present invention relates to an active immunization method for treating tumors containing gangliosides comprising administering to a subject:

(A) an immunogenic effective amount of a lactone of a tumor-associated ganglioside; and

(B) a pharmaceutically acceptable carrier.

In a fourth embodiment, the present invention relates to a method for detecting tumors containing gangliosides comprising:

(A) contacting a test sample with an antibody produced by the process comprising:

(1) immunizing an animal with an immunogenic effective amount of a lactone of a tumor-associated ganglioside and a pharmaceutically acceptable carrier;

(2) isolating the immunized cells from said animal;

(3) fusing the isolated immunized cells with myeloma cells;

(4) screening for hybridomas which produce antibodies having binding specificity to said ganglioside and collecting the antibodies so produced; and

(B) assaying for specific binding of said antibody to antigen in said test sample.

The particular tumor-associated ganglioside employed in the present invention is not critical thereto. Examples of such tumor-associated gangliosides include GD_3 found in melanomas (Pukel, C. S. et al, *J. Exp. Med.*, 155:1133–1147 (1982) and Nudelman, E. et al, *J. Biol. Chem.*, 257:12752–12756 (1982)); GD_2 found in melanomas and neuroectodermal tumors such as neuroblastomas (Cahan, L. et al, *Proc. Natl. Acad. Sci. USA*, 79:7629–7633 (1982)); sialyl Le^a found in gastrointestinal and pancreatic cancers (Magnani, J. L. et al, *J. Biol. Chem.*, 257:14365–14369 (1982)); sialyl Le^X found in colorectal, gastrointestinal and lung adenocarcinomas (Fukushima, K. et al, *Cancer Res.*, 44:5279–5285 (1984)); sialyl difucosyl Le^x found in colorectal, gastrointestinal and lung adenocarcinomas (Fukushi, Y. et al, *J. Biol. Chem.*, 259:10511–10517 (1984)); GM_3 found in melanomas (Taniguchi, M., *Gann*, 75:418–426 (1984); Hirabayashi, Y. et al, *J. Biol. Chem.*, 260:13328–13333 (1985); Nores, G. et al, *J. Immunol.*, 139:3171–3176 (1987); 6C ganglioside found in colorectal carcinomas (Hakomori, S. et al, *Biochem. Biophys. Res. Commun.*, 113:791–798 (1983); G2 ganglioside found in myelogeneous leukemia cells (Fukuda, Y. et al, *J. Biol. Chem.*, 260:1060–1082 (1985); disialosyl Le^a found in colorectal cancers (Nudelman, E. et al, *J. Biol. Chem.*, 261:5487–5495 (1986); monosialyl type 1 chain found in colorectal carcinomas and teratocarcinomas (Nilsson, O. et al, *FEBS Letters*, 182:398–402 (1985); Fakuda, M. N. et al, *J. Biol. Chem.*, 261:5145–5153 (1986); disialosyl type 1 chain found in colorectal cancers (Fukushi, Y. et al, *Biochem.*, 25:2859–2866 (1986); and fucosyl GM_1 found in small cell lung carcinomas (Nilsson, O. et al, *Glycoconjugate J.*, 1:43–49 (1984)).

Lactones of the gangliosides can be prepared by dissolving any ganglioside in glacial acetic acid and allowing the solution to stand for at least 48 hours, followed by lyophilization of the acetic acid. Formation of the ganglioside lactones can be monitored by thin layer chromatography, using high performance thin layer chromatography plates obtained from J. T. Baker Chemical Co. (Phillipsburg, N.J.) and chloroform:methanol:water (50:40:10 (v/v/v)) containing 0.05% (w/v) $CaCl_2$ as a solvent since ganglioside lactones show a distinctively higher mobility than native gangliosides on thin layer chromatography. Note, the above solvent composition is not critical and any well known solvent which can separate gangliosides from the lactones thereof can be employed, for example, as described in Nores, G. A. et al, *J. Immunol.*, 139:3171–3176 (1987).

Alternatively, and more efficiently, ganglioside lactones can be prepared by dissolving any ganglioside in chloroform:methanol:12 N HCl (10:35:4.5 (v/v/v)) and allowing the solution to stand for about one day. The resulting solution is then chromatographed using DEAE-Sephadex in chloroform:methanol:water (0.1:1:1 (v/v/v)). Two main components and several minor components, the structures of the latter remain to be elucidated, are resolvable in this system. The resulting ganglioside lactones can be purified by HPLC on Iatrobeads 6RS8010 in isopropanol:hexane:water (55:25:20 (v/v/v)) with gradient elution being carried out as described by Watanabe, K. et al, J. Lipid Res., 22:1020–1024 (1981). The structure of the purified ganglioside lactones can be verified by direct probe fast atom bombardment mass spectrometry as described in Riboni, L., *J. Biol. Chem.*, 261:8514–8519 (1986).

The particular pharmaceutically acceptable carrier to be used along with the lactone of the tumor-associated ganglioside is not critical to the present invention. Examples of such pharmaceutically acceptable carriers include Bacillus Calmette-Guerin (BCG), diptheria toxoid and tetanus toxoid.

Further, the particular pharmaceutically acceptable carrier to be used along with the antibody produced using the lactones of the tumor-associated gangliosides of the present invention is not critical thereto. Examples of such pharmaceutically acceptable carriers include Bacillus Calmette-Guerin (BCG), diptheria toxoid and tetanus toxoid.

In addition, lactones of tumor-associated gangliosides as immunogens when appropriately assembled in either natural or artificial membranes can be useful as antitumor vaccines (Livingston, P. O. et al, *Proc. Natl. Acad. Sci. USA*, 84:2911–2915 (1987); and Livingston, P. O. et al, *J. Immunol.*, 131:2601–2605 (1983)). Another possible carrier would be a Vaccinia virus in which a specific ganglioside lactone could be assembled (Stott, E. J., *J. Biol.*, 61:3855–3861 (1987); and Hu, S. -L. et al, *J. Biol.*, 62:176–180 (1988)).

A pharmaceutically acceptable diluent can also be employed in the present invention. The particular pharmaceutically acceptable diluent employed is not critical thereto. Examples of such diluents include physiological saline, Ringer's solution, vitamin cocktail and amino acid vitamin cocktail. These diluents can be employed for administering either the lactone of the tumor-associated ganglioside or the antibody having binding specificity thereto.

The lactones of tumor-associated gangliosides may be administered using any of the following modes of administration: intradermal, subcutaneously or intraperitoneal.

The antibodies specific to the tumor-associated gangliosides may be administered intravenously.

The particular animal being immunized with the lactone of the tumor-associated ganglioside is not critical to the present invention. Examples of such animals include mice, rabbits, rats, goats and humans.

As used herein "immunized cells" refers to the sensitized spleen cells of the immunized animal, e.g., those of mice such as Balb/c mice.

The particular myeloma cells employed in the present invention are not critical thereto and can be any well known myeloma cell useful for preparing hybridomas of mouse, rat, rabbit, goat and human origin. Examples of such myeloma cells include HAT sensitive mice myeloma cells such as NS/1 cells and SP-2 cells.

The immunogenic effective amount of the lactone of the tumor-associated ganglioside to be administered in the present invention will vary depending upon the age, weight, sex and species of the animal to be administered. Generally, the immunogenic effective amount is about 2.0 to 5.0 μg, adsorbed on about 20 to 100 μg of carrier per one injection. Generally, from 5 to 10 injections of the ganglioside lactone are employed but the present invention is not limited thereto.

The pharmaceutically effective amount of the antibodies of the present invention to be administered will vary depending upon the age, weight, sex and species of the animal to be administered. Generally, the pharmaceutically effective amount is about 1.0 to 5.0 μg/100 g body weight of animal per one injection. Generally, from 5 to 10 injections of the antibodies are employed but the present invention is not limited thereto.

The particular ganglioside lactone or antibody thereto which will be administered will depend upon the particular ganglioside lactone present in the tumor which is intended to be treated. Information as to the particular ganglioside present in the tumor can be obtained by a serum assay or biopsy assay for the various gangliosides. As used herein, "treatment" means both prevention of tumor formation and treatment of existing tumors.

Immunizing the animals, e.g., mice, with the ganglioside lactones of the present invention, isolating the immunized cells, fusing the immunized cells with, e.g., mouse myeloma cells, and culturing the resulting fused cells under conditions which allow for growth of hybridomas, are all conducted by methods well known and readily determined in the art (Young, W. W. et al, *J. Exp. Med.*, 150:1008–1019 (1979) and Fukushi, Y. et al, *J. Biol. Chem.*, 259:4681–4685 (1984)).

The resulting hybridomas are then screened so as to isolate those which produce monoclonal antibodies having binding specificity to the ganglioside lactones, in for example a solid-phase radioimmunoassay using ganglioside-coated wells and assaying using a second antibody (rabbit anti-mouse IgM+IgG (Miles Biochemical, Elkhart, Ind.)) and ^{125}I-labeled Protein A as described in more detail hereinafter.

In the method for detecting tumors containing gangliosides of the present invention, "test sample" means, for example, tissue biopsies, serum, ascites fluid and spinal fluid.

In this method, detection can occur either in vitro or in vivo. In vitro detection can be carried out using any of the well known in vitro immunological assays, such as those described by Young, W. W. et al, *J. Exp. Med.*, 150:1008–1019 (1979) and Kannagi, R. et al, *Cancer Res.*, 43:4997–5005 (1983). Further, in vivo detection can be carried out using any of the well known in vivo immunological assays, such as those described by Burchell, J. et al, *Int. J. Cancer*, 34:763–768 (1984); Epenetos, A. A. et al, *Lancet*, 2:999–1004 (1982); Chatal, J. F. et al, *J. Nuclear Med.*, 25:307–314 (1984); Munz, D. L. et al, *J. Nuclear Med.*, 27:1739–1745 (1986); and Keenan, A. N. et al, *J. Nuclear Med.*, 26:531–537 (1985).

The following examples are provided for illustrative purposes only and are in no way intended to limit the scope of the present invention.

EXAMPLE 1

Method of Preparation of Monoclonal Antibodies Using Ganglioside Lactones

A. Production of Monoclonal Antibodies

GM_3 was extracted from dog erythrocytes by isopropanol-hexane-water (55:25:20 (v/v/v)) and separated by Folch partition followed by ion exchange chromatography as described in Hakomori, S., In *Handbook of Lipid Research*, Vol. 3, Sphingolipid Biochemistry (Kanfer, J. N. et al Eds., Plenum, N.Y., pages 1–165 (1983), Plenum Publishing, New York, pages 1–165 (1983)).

40 μg of GM_3 or 40 μg GM_3 lactone, prepared from the resulting GM_3 using acetic acid or the chloroform-methanol-HCl method as described above, was suspended in 4.0 ml of distilled water, sonicated, and mixed with 1.0 mg of acid-treated *Salmonella minnesota*, as described by Young, W. W. et al, *J. Exp. Med.*, 150:1008–1019 (1979). The *Salmonella minnesota* used was suspended in 1.0% (v/v) aqueous acetic acid and heated for 1 hour at 80° C., followed by dialysis and lyophilization. The suspension was then incubated for 10 min at 37° C. and lyophilized. The lyophilized material was resuspended in 4.0 ml of PBS and aliquots of 2.0 μg of GM_3 or GM_3 lactone on 50 μg of *Salmonella minnesota* were injected intravenously weekly into BALB/c mice. A total of 8 injections were made. Three days after the last booster injection, 10^8 spleen cells from the mice were harvested and fused with 5×10^7 mouse myeloma SP2 cells as described by Young, W. W. et al, *J. Exp. Med.*, 150:1008–1019 (1979). The resulting hybridomas were grown in RPMI medium supplemented with 10% (v/v) fetal calf serum, as described in detail in Young, W. W. et al, *J. Exp. Med.*, 150:1008–1019 (1979).

The culture supernatants of the resulting hybridomas on the seventh day after fusion were screened on 96-well plastic plates (Becton-Dickinson, Oxnard, Calif.) which had been pre-coated with 0.1% (w/v) gelatin in a solid-phase radioimmunoassay. More specifically, the gelatin-coated plates were incubated with 200 μl of 0.1% (w/v) bovine serum albumin for 24 hours at 4° C., washed with PBS once and incubated with 50 μl of a 0.2 μmole/ml GM_3 or GM_3 lactone in PBS solution overnight at room temperature. The wells were then washed with PBS, and culture supernatants from the hybridomas as the first antibody were added and incubated for 2 hours at room temperature. Then, the first antibody bound to each ganglioside-coated well was assayed using 50 μl of a second antibody (rabbit anti-mouse IgM+IgG (Miles Biochemical, Elkhart, Ind.)) and 50 μl of ^{125}I-labeled Protein A to detect binding of the second antibody to the first antibody. Each well was cut and the radioactivity counted in a gamma counter. Only strongly active wells (greater than 2,000 cpm) were regarded as positive. The results are shown in Table I below.

TABLE I

Comparison of Immunogenicity of Native GM_3 and GM_3 Lactone to BALB/c Mice	Immunization with:			
	GM_3 Lactone		GM_3	
	strong positive (2000 cpm)	weak positive (800–1500 cpm)	strong positive (2000 cpm)	weak positive (800–1500 cpm)
Reactivity with GM_3 lactone	1/192; 5/288*; 2/288*	23/192	0/192	5/192
Reactivity with GM_3	0/192	9/192	0/192	0/192

*Separate experiments based on three 96-well plates. Antibodies from these hybridomas react with both GM_3 and GM_3 lactone after their establishment.

As shown in Table I above, in one experiment, after immunization with GM_3 lactone, 7 strongly positive hybridomas were obtained out of 288 clones screened. On the other hand, no hybridomas were obtained after immunization with native GM_3 and screening of 192 clones. This difference is much greater if the 23 weakly positive hybridomas obtained after immunization with GM_3 lactone are included.

The results in Table I above demonstrate that immunization of mice with GM_3 lactone, but not with native GM_3, elicits many bybridomas secreting monoclonal antibodies. These results demonstrate that GM_3 lactone is a superior immunogen than native GM_3.

One of the monoclonal antibodies established after immunization of mice with GM_3 lactone was designated DH2antibody. The isotope of this antibody, determined using rabbit anti-mouse IgG antibodies (Miles Biochemical, Elkhart, Ind.) was identified as IgG_3. DH2 antibody was saved for further analysis. Hybridoma DH2 has been deposited with the American Type Culture Collection under accession number HB 9663.

B. Analysis of DH2 Antibody

In order to determine the reactivity of DH2antibody to GM_3 and GM_3 lactone, the following experiments were carried out.

20 pmole of GM_3 (.) or GM_3 lactone (o) was added along with 50 ng PC and 30 ng cholesterol, dissolved in ethanol, per well of 96-well polyvinyl plastic plates and dried (see FIG. 2A) or 20 pmole of GM_3 (.) or GM_3 lactone (o) was dissolved in PBS per well of 96-well gelatin-coated polyvinyl plastic plates and dried (see FIG. 2B). The wells were blocked with 5.0% (w/v) bovine serum albumin in PBS for 2 hours and reacted with the various concentrations of DH2 antibody shown in FIGS. 2A and 2B for 2 hours at room temperature. After washing, bound antibody was detected using 50 μl of a second antibody (rabbit anti-mouse IgG and IgM antibody (Miles Biochemical, Elkhart, Ind.)) followed by detection with 50 μl of ^{125}I-Protein A. Finally, the wells were cut and the radioactivity was counted in a gamma counter. The results are shown in FIGS. 2A and 2B.

As shown in FIG. 2B, DH2 antibody reacted with GM_3 lactone preferentially, but also reacted with GM_3, when GM_3 lactone and GM_3 were coated on gelatin-coated polyvinyl plastic plates. However, as shown in FIG. 2A, DH2 antibody did not show reactivity with GM_3 lactone dried from an ethanol solution, i.e., only GM_3 strongly reacted with DH2 antibody when dried from an ethanol solution. This property is characteristic of anti-ganglioside antibodies established after immunization with ganglioside lactones, i.e., anti-ganglioside lactone antibodies cross-react strongly only with native gangliosides when native gangliosides are coated on either a polyvinyl plastic surface or present on a lipid bi-layer. However, anti-ganglioside lactone antibodies react strongly with both lactones and native gangliosides when coated on gelatin-coated polyvinyl plastic plates. Lactones may have special conformation, which causes them to adhere on a polyvinyl plastic surface through their hydrophobic epitope. Therefore, lactones directly coated on a polyvinyl plastic surface show a very weak reactivity with specific antibodies, whereas lactones do not adhere on gelatin through the hydrophobic epitope but, rather, interact through the ceramide moiety. Thus, it is necessary, that lactones be presented on gelatin-coated polyvinyl plastic plates in order to demonstrate their reactivity.

In order to determine the reactivity of DH2 antibody to glycolipids other than GM_3 or GM_3 lactone, the following experiments were carried out.

20 pmole of each of the glycolipids shown in FIG. 3 were added separately along with 50 ng PC and 30 ng cholesterol, dissolved in ethanol, per well of 96-well polyvinyl plastic plates and dried. The binding of DH2 antibody to the wells was carried out as described above. The results are shown in FIG. 3.

As shown in FIG. 3, DH2 antibody reacted strongly with GM_3 containing N-acetyl-neuraminic acid ($NeuAcGM_3$ (.)), but only weakly with GM_3 containing N-glycolyl-neuraminic acid ($NeuGcGM_3$) (o) or sialylparagloboside (SPG (■)). Also as shown in FIG. 3, DH2 antibody did not react with the other glycolipids tested, i.e., GM_1, GD1a, GD1b, GT1, galactosylceramide and sialyllactonorhexaosylceramide (□).

The specificity of DH2 antibody for glycolipids was further determined by thin layer chromatography immunostaining on Baker's thin layer chromatography plates (J. T. Baker Chemical Co., Phillipsburg, N.J.) using a slightly modified version of the procedure described by Magnani, J. L. et al, *Anal. Biochem.*, 109:399–402 (1980). More specifically, glycolipids were applied on the thin layer chromatography plates using a solvent system of chloroform:methanol;water ((50:40:10) (v/v/v)) containing 0.05% (w/v) $CaCl_2$. After drying, the thin layer chromatography plates were blocked for 2 hours with 5.0% (w/v) bovine serum albumin in PBS and reacted by immersion in DH2 hybridoma culture supernatant at room temperature overnight. After washing, bound antibody was detected using 50 μl of a second antibody (rabbit anti-mouse IgG antibody (Miles Biochemical, Elkhart, IN)), followed by detection with 50 μl of ^{125}I-Protein A. The thin layer chromatography plates were assayed by autoradiography. The results are shown in Table II below.

TABLE II

Specificity of DH2 Antibody for Glycolipids Determined by Thin Layer Chromatography Immunostaining

Glycolipid	Reactivity
$NeuAcGM_3$ (dog erythrocytes)	+
$NeuAcGM_3$ (B16 melanoma)	+
$NeuAcGM_3$ (rat brain)	+
$NeuAcGM_3$ lactone (dog erythrocytes)	+
$NeuGM_3$	−
$NeuGcGM_3$ lactone	−
Sialylparagloboside	±
Sialylparagloboside lactone	−
Sialyllactonorhexaosylceramide	−
Sialyllactonorhexaosylceramide lactone	−
$NeuAcGM_3$ ethylester	−
$NeuAcGM_3$ gangliosidol	−

TABLE II-continued

Specificity of DH2 Antibody for Glycolipids Determined by Thin Layer Chromatography Immunostaining	
Glycolipid	Reactivity
GM_2	−
GM_1	−
GM_1 lactone	−
GD1a	−
GD1b	−
GD1b lactone	−
GT1	−
Asialo GM_2	−
Lactosylceramide	−
Glucosylceramide	−

+, positive reactivity
±, weak positive reactivity
−, negative reactivity

As shown in Table II above, DH2 antibody reacted strongly only with GM_3, which contains N-acetylneuraminic acid (NeuAc), and its lactone, not with any of the other glycolipids tested. Further, weak staining was observed with SPG, but the lactone of SPG was not reactive. It is noteworthy that GM_3 ethylester (NeuAcGM_3 ethylester) and the reduced form of GM_3, in which the carboxyl group of the sialic acid was reduced to alcohol (NeuAcGM_3 gangliosidol), were not reactive. NeuAcGM_3 gangliosidol has no carboxyl group. Instead it has a hydroxyl group at the C-1 position of the sialic acid. Thus, it has an entirely different conformational structure from GM_3 and cannot be converted into a lactone. Further, since various types of lactones derived from other gangliosides, such as lactones of SPG, sialyllactonorhexaosylceramide, GM_1, and GD1b were all negative, DH2 antibody reactivity to lactone was limited to that of N-acetyl GM_3. These results demonstrate that DH2 antibody reacts with both GM_3 and GM_3 lactone but, not with other types of gangliosides or other lactones. These results also demonstrate that DH2 antibody shows preferential reactivity with GM_3 lactone under certain conditions, i.e., when the GM_3 lactone is dried on a gelatin or BSA coated polyvinyl plastic surface; and preferential reactivity with GM_3 under other conditions, i.e., when the GM_3 lactone is directly dried from an ethanol solution on a polyvinyl plastic surface.

In order to compare the reactivity of DH2 antibody to various cell lines in comparison with that of M2590 antibody, an IgM monoclonal antibody established after immunization of C57BL/6 mice with B16 melanoma cells as described in Taniguchi, M., *Jpn. J. Cancer Chemother.*, 75:413–426 (1984), the following experiments were carried out.

Various myeloma and other tumor cell lines shown in Table III below were harvested using 0.2% (w/v) EDTA and 0.2% (w/v) trypsin, washed with PBS and incubated with 20 μg/ml of DH2 antibody or 10 μg/ml of M2590 antibody, as first antibodies, for 1 hour in ice. After several washes with ice cold PBS, the cell lines were incubated with 50 μl of fluorescinlableded goat anti-mouse IgG+IgM (Miles Biochemical, Elkhart, Ind.) as a second antibody and immunofluorescence was analyzed by microscopy, using, as negative control cells, cells which had been incubated with the second antibodies but without the first antibodies. The results are shown in Table III below.

TABLE III

Immunofluorescence Test of various Cell Lines With DH2 and M2590 Antibodies

Cell Line	Origin	Reactivity with DH2	Reactivity with M2590
B16	mouse melanoma	+++	+++
B16F1	mouse melanoma	+++	+++
B16F10	mouse melanoma	+++	+++
M2669	human melanoma	+	+
M1733	human melanoma	+	+
M2981	human melanoma	−	±
M2291	human melanoma	−	ND
P36-F4	human melanoma	+	−
HMV-I491B10	human melanoma	−	−
FM3A/F28-7	mouse breast carcinoma	−	−
FUA169	mouse breast carcinoma	++	++
HTB19	human lung carcinoma	±	ND
A431	human epidermoid carcinoma	−	−
SW403	humun colon carcinoma	−	−
MKN45	human gastric carcinoma	−	−
K562	human erythroleukemia	−	ND
A-204	human rhabdomyosarcoma	−	−
BHK	hamster fibroblasts	±	+
NRK	rat fibroblasts	+	+
FRE	rat fibroblasts	+	+
dog erythrocytes		+++	+++
rabbit erythrocytes		−	−
human erythrocytes		−	−

+++, almost 100% of the cells were positive; ++, more than 50% of the cells were positive; +, less than 50% of the cells were positive; ±, less than 1% of the cells were positive; −, negative; ND, not determined.

As shown in Table III above, those cells showing strong immunofluorescence with DH2 antibody and M2590 antibody were B16 mouse melanoma and its variants, mouse breast carcinoma FUA169 and dog erythrocytes. All of these highly reactive cells have been characterized by a relatively high concentration of GM_3. On the other hand, as shown in Table III above, normal cells or non-melanoma cells, which contain a relatively low concentration of GM_3, did not react with DH2 antibody. These results demonstrate that DH2 antibody can recognize the density of GM_3 organized in the cell surface membrane, i.e., DH2 antibody can only react with GM_3 at densities higher than a threshold value of about 10–15 mol %. In this respect, DH2 antibody's specificity is similar to that of M2590 antibody.

EXAMPLE 2

Effective DH2 Antibody on B16 Melanoma Cell Growth In Vitro and In Vivo

A. In Vitro Study

To study the effect of DH2 antibody on B16 melanoma cell growth in vitro, B16 melanoma cells were harvested with 0.2% (w/v) EDTA and 0.2% (w/v) trypsin and placed in 24 well culture plates (Becton-Dickinson, Oxnard, Calif.) at a density of 5×10^4 cells/-well and grown in RPMI medium supplemented with 3.0% (v/v) fetal calf serum at 37° C. After 24 hours and 48 hours, 50 μg/ml of DH2 antibody (.); 50 μg/ml of M2590 antibody (▲), which, as discussed above, is an IgM antibody which is also directed to GM_3 and GM_3 lactone; 50 μg/ml of CU-1 anti-Tn (■), which is an IgG_3 antibody which reacts with Tn-antigens; or PBS for control (o) was added. The number of cells were counted at 24 hours, 43 hours, 55 hours and 72 hours after the beginning of culturing. The results are shown in FIG. 4.

As shown in FIG. 4, cell growth of b16 melanoma was greatly inhibited by the presence of DH2 antibody when compared to M2590 antibody and CU-1 anti-Tn.

In a similar experiment, using human colonic carcinoma cell line SW403, which does not express GM_3, inhibition of human colonic carcinoma cell growth was not observed using DH2 antibody. These results demonstrate that DH2 antibody, orginally raised after immunization with GM_3 lactone, is capable of inhibiting melanoma growth in vitro.

Furthermore, as shown in FIG. 5, wherein the effects of the following concentrations of DH2 antibody on B16 melanoma cell growth in vitro was ascertained: 100 μg/ml (.); 50 μg/ml (■); 25 μg/ml (o); and 12.5 μg/ml (Δ); and PBS as a control (□) was carried out as described above, the cell growth inhibition induced by DH2 antibody is dose-dependent, i.e., clear inhibition is only observed at high concentrations of antibody (50–100 μg/ml).

The inhibition of B16 melanoma cell growth caused by DH2 antibody can be reversed if the cells are exposed to normal media without DH2 antibody.

B. In Vivo Study

To study the effect of DH2 antibody on B16 melanoma cell growth in vivo, two groups of four C57BL/6 mice were given subcutaneous injections of 5×10^6 cells of B16 melanoma at each of two separated sites on the back (day 0). On days 0, 2, 4, 6, 8, 10, 12 and 14, experimental group animals were injected with 4.0 μg of DH2 antibody in 400 μl of PBS via the tail vein. Control group animals were injected with 400 μl of PBS on the same day. Three diameters (d_1, d_2 and d_3) of the tumors were measured and the tumor volumes were calculated by the formula $(\pi/2)(d_1, d_2, d_3)$. The results are shown in FIGS. 6A and 6B.

As shown in FIG. 6A, DH2 antibody exhibits significant growth inhibition of B16 melanoma cells in vivo. More specifically, in 2 out of the 8 cases of B16 melanoma cells in mice, B16 melanoma cell growth was almost completely inhibited until day 25. Control animals, shown in FIG. 6B, all died before day 20. The average life-span of B16 melanoma-bearing mice treated with DH2 antibody was 22.5 days, while that of control animals was 12.5 days.

DH2 antibody distribution was determined in B16 melanoma-bearing mice after injection of ^{125}I-labeled DH2 antibody. More specifically, three C57BL/6 mice were injected with 5×10^6 B16 melanoma cells subcutaneously. Drinking water for the mice was changed to 0.1% (w/v) KI 5 days before DH2 antibody injection. 10 days after B16 melanoma cell innoculation, 20 μg (60 μCi) of ^{125}I-labeled-DH2 antibody prepared using IODO-BEADS (Pierce Chemical, Rockford, Ill.) were injected via the tail vein and mice were sacrificed 72 hours later. After taking a blood sample from the cardiac cavity, PBS was injected into the heart to flush blood from the tissues. Samples from tissues and tumors were weighed and the radioactivity was counted in a gamma counter. The in vivo tissue distribution was expressed as a ratio of radioactivity in tumor to normal tissues (cpm/g in tumor tissue)/(cpm/g in normal tissue)). The results are shown in Table IV below.

TABLE IV

Distribution of ^{125}I-labeled DH2 Antibody in Tissues of B16 Melanoma Bearing Mice

Organ or Tissue	(cpm/g of tumor tissue)/ (cpm/g of normal tissue) (mean value from triplicate experiments)
Blood	1.04
Bone marrow	42.00
Thymus	14.10
Spleen	10.00
Skin	5.55
Muscle	13.51
Bone	14.62
Heart muscle	7.81
Thyroid and adjacent tissue	6.11
Lung	4.79
Liver	14.53
Kidney	4.13
Intestine	12.32
Intestinal mesentery	14.21
Brain	90.60
Urinary bladder	3.94
Uterus and attached tissue	4.44

As shown in Table IV above, the highest level of activity was observed in the original melanomas subcutaneously grown and in blood samples, followed by urogenital tissue. The lowest activity was found in bone marrow and the brain. These results demonstrate that DH2 antibody strongly binds to melanoma cells in vivo as well as to blood, although other tissues and organs showed much less binding activity than the melanoma cells.

EXAMPLE 3

Cytotoxicity Induced by DH2 Antibody

The effects of DH2 antibody on antibodydependent cytotoxicity was studied using the 4 hour chromium assay described by Grabstein, K. In *Selected Release Methods of Cellular Immunology*, Mishell, B. B. et al, Eds., pages 124–137, Freeman & Co., San Francisco (1980). More specifically, mononuclear cells from peripheral blood from healthy human donors prepared by Ficoll-Paque (Pharmacia, Piscataway, N.J.) or lymphocytes harvested from spleens of C57BL/6 mice, were used as effector cells. 1.0×10^6 B16 melanoma cells were used as target cells and labeled for 2 hours with 100 μCi sodium (^{51}Cr) chromate in RPMI medium supplemented with 3.0% (v/v) fetal calf serum at 37° C. in a CO_2 incubator, washed, incubated with 50 μg/ml of DH2 antibody in RPMI medium supplemented with 3.0% (v/v) fetal calf serum for 30 min at 37° C. in a CO_2 incubator and washed again. ^{51}Cr-labeled B16 melanoma cells treated with DH2 antibody were placed in 96-well round bottom plates (Costar, Cambridge, Mass.) at a density of 5×10^3 cells/well, and incubated with various concentrations of effector cells as shown in Table V below for 4 hours at 37° C. The plates were then centrifuged at 500× g for 5 min and the radioactivity was measured in a 125 μl aliguot of each supernatant using a gamma counter.

Spontaneous ^{51}Cr release was determined in wells that contained only labeled B16 melanoma cells treated with DH2 antibody for 24 hours.

Total release was determined using the supernatant of the wells in which the cells were lysed with 2.0% (v/v) Triton X-100 and centrifuged. The percentage of lysis was calculated as follows:

$$\frac{(\text{Experimental release}) - (\text{Spontaneous release})}{(\text{Total release}) - (\text{Spontaneous release})} \times 100$$

The results are shown in Table V below.

TABLE V

Antibody-dependent Cytotoxicity of DH2 Antibody Against B16 Melanoma Cells

	Human effector cells				C57BL/6 mouse effector cells			
Effector: target ratio	200	100	50	25	200	100	50	25
Percentage of lysis	20.3	7.0	0	0	12.3	6.3	2.9	5.3

As Table V above clearly demonstrates, antibody-dependent cytotoxicity was demonstrated by a lysis of the target cells at high effector:target ratio. This lysis was observed with both human and mouse effector cells. The release of ^{51}Cr observed in this experiment was found to be due to lysis of target cells by cytotoxic effector cells, since DH2 antibody alone did not cause significant release of ^{51}Cr under the same conditions, i.e., release of ^{51}Cr by DH2 antibody alone during 24 hours was only 3.0%. These results demonstrate that DH2 antibody shows a clear antibody-dependent cytotoxic effect on melanoma cells.

EXAMPLE 4

Active Immunization With GM_3 Lactone

In order to determine the effect on B16 melanoma cell growth by active immunization of mice with GM_3 lactone or GM_3 coated on acid-treated *Salmonella minnesota* the following experiments were carried out.

10 BALB/c mice were immunized with native GM_3 or GM_3 lactone coated on acid-treated *Salmonella minnesota* as described above. Immunization was carried out by intravenous injection of 200 μl of the GM_3 or GM_3 lactone preparation once per week for 4 weeks. Subsequently, 1.0×10^5 B16 melanoma cells of clones F-1 or F-10, were subcutaneously injected into the back of the mice and tumor growth was observed after 20 days. As controls, other glycolipids, such as paragloboside coated on acid-treated *Salmonella minnesota*, and *Salmonella minnesota* alone, were used in the same amounts as discussed above. The results are shown in Table VI below.

TABLE VI

Effect of Immunization With GM_3 Lactone on B16 Melanoma Development

	Salmonella minnesota alone	GM_3, Adsorbed on *Salmonella minnesota*	GM_3 Lactone Adsorbed on *Salmonella minnesota*
Melanoma B16			
F-1	10/10	10/10	2/10
F-10	10/10	10/10	3/10

In Table VI above, the numbers indicate the number of animals which died over the total number of animals immunized. The results in Table VI above demonstrate that tumor growth was reduced in the group immunized with GM_3 lactone but not in the group immunized with GM_3 or with other glycolipids, such as paragloboside coated on *Salmonella minnesota*, or with *Salmonella minnesota* alone. These results demonstrate that GM_3 lactone but not GM_3 is capable of suppressing tumor growth in vivo.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

I claim:

1. A method for the production of antibodies to a tumor-associated ganglioside comprising:
(1) immunizing an animal with an immunogenic amount of a lactone of a tumor-associated ganglioside and a pharmaceutically acceptable carrier;
(2) isolating the immunized cells from said animal;
(3) fusing the isolated immunized cells with myeloma cells; and
(4) screening for hybridomas which produce antibodies having binding specificity to said ganglioside and collecting the antibodies so produced.

2. The method as claimed in claim 1, wherein said tumor-associated ganglioside is selected from the group consisting of GD_3, GD_2, sialyl Le^a, sialyl Le^X, sialyl difucosyl Le^x, GM_3, 6C ganglioside, G2 ganglioside, disialosyl Le^a, monosialyl type 1 chain, disialosyl type 1 chain and fucosyl GM_1.

3. The method as claimed in claim 1, wherein said immunogenic amount is about 2.0 to 5.0 μg adsorbed on about 20 to 100 μg of carrier.

4. The method as claimed in claim 1, wherein said pharmaceutically acceptable carrier is selected from the group consisting of Bacillus Calmette-Guerin (BCG), diptheria toxoid, and tetanus toxoid.

5. The method as claimed in claim 1, wherein said pharmaceutically acceptable carrier is selected from the group consisting of an artificial membrane a natural membrane and vaccinia virus.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,389,530

DATED : February 14, 1995

INVENTOR(S) : Sen-itiroh HAKOMORI

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Col. 4, line 11 delete "(●)" and insert -- (■) --.

Col. 10, line 63 delete "$NeuGM_3$" and insert -- $NeuGcGM_3$ --.

Signed and Sealed this

Twentieth Day of February, 1996

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks